ORIGINAL ARTICLE

Multi-approach comparative study of the two most prevalent genotypes of pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae) in Chile

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Abstract

The pea aphid, *Acyrthosiphon pisum* Harris, is able to colonize various habitats and form genetically distinct biotypes worldwide. In Chile, few genotypes are dominating in space and time and are qualified as "superclones". Here, we compared in the laboratory different biological features of the most common clones of *A. pisum* collected from pea (Ap1) and alfalfa (Ap2) fields, in order to gain some insight on their differential ability to colonize broad bean plants. We also studied their probing behavior, profiled their proteome and characterized their community of endosymbionts. We found that the Ap1 genotype performed better on *Vicia faba* and realized more salivation events in phloem than Ap2. In addition, Ap1 presented a higher prevalence of the endosymbiont *Serratia symbiotica*. Among the total of 40 proteins that were differentially expressed, 14 and 26 were upregulated in Ap1 and Ap2 genotypes, respectively. The symbionin from *Buchnera aphidicola* was found to be upregulated in Ap1. A field experiment showed that both genotypes were able to colonize wild legumes, with Ap1 reproducing better on *Vicia nigricans* than Ap2. However, Ap2 exhibited higher reproduction in the other three wild legumes, suggesting higher invasiveness capacity on wild plants. Variation in the ability to colonize, feeding behavior and the putative involvement of differentially regulated proteins between Ap1 and Ap2 are discussed in relation to their respective endosymbiotic composition, nutritional lifestyle and consequences on their "superclone" status.

Key words: 2D-DIGE, EPG, feeding behavior, population growth rate, proteomics, secondary endosymbiont, superclone.

INTRODUCTION

The pea aphid (*Acyrthosiphon pisum* Harris) is known as a pest of significant economic importance with a worldwide distribution (Blackman & Eastop 2000). It exhibits populations specialized on different wild and cultivated species belonging to the Fabaceae family, such as *Pisum* sp., *Vicia* sp., *Phaseolus* sp., *Medicago*

sp. and *Trifolium* sp. (Eastop 1971; Ferrari *et al*. 2006; Blackman & Eastop 2007; Turpeau-Ait Ighil *et al*. 2011). This species shows large phenotypic variability depending on the environment (Frantz *et al*. 2009). Indeed, during the last millennia, the pea aphid showed a rapid genetic diversification involving host plant shifts, which led to sympatric populations on legume crops as well as wild plants of the same botanical family (Peccoud *et al*. 2009a; Schwarzkopf *et al*. 2013). The expansion of potential hosts was probably partly influenced by global warming and anthropogenic factors (Peccoud *et al*. 2008). According to a study undertaken by the French National Institute for Agriculture Research, based on genetic markers and tests of host plant specificity, at least 11 biotypes of

A. pisum have been recognized as being adapted to different legume host plants in Western Europe (Peccoud *et al.* 2009b; Turpeau-Ait Ighil *et al.* 2011). In particular, the existence of three distinct genotypes, constituted by populations found on pea and broad bean, alfalfa and red clover, was confirmed by phenotypic and genetic analyses (Simon *et al.* 2003; Frantz *et al.* 2009).

Acyrthosiphon pisum was first reported in central Chile 40 years ago (Rojas 2005). Phenotypic and genotypic analyses confirmed their strong host specialization and demonstrated parthenogenesis as their only reproductive mode. Interestingly, genotypes were found to correspond to host-specialized populations from the Old World, indicating that clones descended from particular Eurasian biotypes, which apparently involved at least three successful introduction events followed by spread on different crops (Peccoud *et al.* 2008). This ecological specialization and reproductive mode are two key traits that contribute to aphid invasiveness, particularly in *A. pisum* (Figueroa *et al.* 2018). In particular, three genotypes are predominant – Ap1, Ap2 and Ap3 – and specialized on pea (*Pisum sativum* L.), alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), respectively (Peccoud *et al.* 2008; Lu *et al.* 2016). As shown by Peccoud *et al.* (2008), Chilean genotypes Ap1, Ap2 and Ap3 could be associated with European individuals from pea, alfalfa and red clover races, respectively. The genotype Ap1 shows a wider host range in Chile, by settling on nine different host plants, with large populations on pea, lentils and broad bean plantations. In contrast, genotype Ap2 was found on seven host plants, mostly concentrated on alfalfa plantations, including broad bean. The clone Ap3 was found on six host plants, mostly on red clover plantations, but also on broad bean. In addition, Ap1 clone has been found hosting on more species of wild legume than the Ap2 clone. This pattern suggests that the most frequent clone Ap1 has a stronger invasive potential than Ap2 or Ap3 (Peccoud *et al.* 2008). However, the comparison between these clones, in terms of their ability to colonize, feed and display physiological variation, has not been established yet.

In this study, the phenotypic differences between the two most common clones of *A. pisum* in Chile were further investigated in order to reveal the mechanisms involved in the variation in invasiveness among these genotypes. Specifically, we sampled pea aphids from pea and alfalfa fields and compared, in the laboratory, their ability to colonize, their feeding behavior, their symbiont community and their whole-body proteomic profile on a common plant. We complemented our study with a field experiment evaluating their ability to colonize wild legumes in the field.

MATERIALS AND METHODS

Aphid and plant

Colonies of the pea aphid *A. pisum* were originated from single females collected from a pea (*P. sativum*) field and alfalfa (*M. sativa*) field in the Maule region, Chile. Laboratory mass rearing was undertaken on broad bean plants (*Vicia faba* L. cv. "Anka Mapu") in the Laboratorio Interacciones Insecto-Planta for several years (University of Talca, Chile). Colonies were maintained at constant temperature (20 ± 2°C), relative humidity (60 ± 10%) and photoperiod (16 h light : 8 h dark) in rearing chambers.

Microsatellite genotyping and endosymbiont screening

First, microsatellite genotyping of both pea aphid clones collected was carried out to verify that samples were genetically different and to check their assignment to pea and alfalfa races. Ten individual aphids (wingless) were taken at random from the stock colony of each clone and DNA extraction from individual aphids and polymerase chain reaction (PCR) conditions were used following Peccoud *et al.* (2008) with minor modifications. Genotyping was carried out using the M13 labeling technique with fluorescent dyes described by Schuelke (2000), followed by automated fragment analysis by Macrogen. (Seoul, Korea). Eight microsatellite loci specific to *A. pisum* were used: *AlB04M*, *AlB07M*, *AlB08M*, *AlB12M*, *Ap03M*, *ApF08M*, *ApH08M* and *ApH10M* (Peccoud *et al.* 2008, 2009b). Allele sizes were determined using GENEMARK version 1.3 (Borodovsky & McIninch 1993). Endosymbiont detection in the aphids was run in order to check the genotype identity of clones and the endosymbionts harbored by the aphids before the study. For this, the procedure described by Peccoud *et al.* (2014) was followed, using two multiplex PCRs, on the same whole-body DNA extracts than the ones used for genotyping. Screened endosymbionts were *Buchnera aphidicola*, *Spiroplasma* sp., *Regiella insecticola*, *Hamiltonella defensa*, *Rickettsiella* sp., pea aphid X-type symbiont (PAXS), *Serratia symbiotica* and *Rickettsia* sp.. The process was repeated after the study to confirm the persistence of the genetic identity and of endosymbiont profiles.

Ability to colonize in the laboratory

Age-synchronized nymphs of each clone were separately placed on potted plants of *V. faba* (2-week-old plants, stage 12 on the BBCH-scale), and 2 weeks after, the number of nymph and adult aphids was counted. In order to check whether the initial density would affect the population growth rate, the experiment was carried out with two initial nymphs and with four initial nymphs. The aphid-hosting aphids were cultivated in plastic pots filled with a mixture of vermiculite and perlite (50:50) in a climate-controlled room (16 h light; $20 \pm 2°C$; $60 \pm 10\%$ relative humidity). Each treatment was composed of 20 replicates. The population growth rate was calculated for each treatment as $(Ln(Nf) - Ln(Ni))/(tf - ti)$, where Ni and Nf indicate the initial and final number of aphids, respectively, and $(tf - ti)$ corresponds to the period (in days) between the beginning and the end of the experiment (Turcotte *et al*. 2011). The Mann–Whitney non-parametric method was used to compare the means for each treatment.

Feeding behavior

In order to check for any behavioral adaptation for better performance on host plants, stylets probing of both genotypes was recorded with a Giga-4d DC-Electrical Penetration Graph (EPG) (EPG Systems, Wageningen, The Netherlands). Aphids were glued to a 25-μm-thin gold wire (EPG Systems) with conductive silver paint (PELCO Colloidal Silver No 16031; Ted Pella, Redding, CA, USA), attached to the EPG probe, and then placed on *V. faba* leaves. An electrode put into the soil closed the circuit. Voltage fluctuations generated particular waveforms according to the different probing phases (non-probing, intercellular penetration, intracellular puncture, saliva secretion and xylem/phloem ingestion). The EPG waveforms were recorded using Stylet+d software (EPG Systems) and analyzed using A2EPG software (Adasme-Carreño *et al*. 2015). Sequential and non-sequential parameters were calculated by an Excel workbook of Sarria *et al*. (2009) and were compared between the two genotypes by multivariate ANOVA. For each clone, individuals (n = 10 and 12 for pea and alfalfa genotypes, respectively) were recorded during 8 h.

Sample preparation for 2D-DIGE

To compare their proteomic profiles, 100 mg adult aphids of each clone, directly sampled from the rearing on *V. faba*, were ground in UT buffer (7 M urea, 2 M thiourea, 0.5% (w/v) CHAPS). Proteins from each sample were then extracted according to a conventional trichloroacetic acid protocol. Ground samples were centrifuged at 4°C at 12,000 g for 10 min. The supernatants were collected and 25 μL trichloroacetic acid (100%) was added. After 30 min of incubation on ice, samples were centrifuged for 5 min at 4°C at 15,000 g. The pellets were kept and washed twice with acetone (100%). The pellets resulting from protein precipitation were resuspended in rehydration buffer (6 M urea, 2 M thiourea, 10% (w/v) CHAPS, 1% (w/v) ASB14 and 30 M Tris, pH 8.5). Quantification of total proteins was carried out using the RC-DC protein assay (Bio-Rad, Hercules, CA, USA). Samples for fluorescent labeling were resuspended in the rehydration buffer, and the pH was adjusted to 8.5 with 100 mM NaOH. Proteins were labeled with the cyanine dye Cy2, Cy3 or Cy5 according to the manufacturer's instructions (Lumiprobe, Hannover, Germany), in order to obtain four technical replicates per clone. A conventional dye swap for difference gel electrophoresis (DIGE) was carried out. Two protein samples corresponding to the two genotypes were labeled either with Cy3 or Cy5. A combined Cy2-labeled internal standard containing equal amounts of all protein extracts was included in every gel. Analytical 2D gels containing CyDye-labeled samples (25 μg Cy2-, Cy3- and Cy5-labeled protein) were used for quantitative analysis and preparative gel containing non-labeled samples (225 μg protein from each aphid clone) was used for picking. The volume of each mix of labeled proteins was adjusted with UT-Tris buffer to 225 μL and completed with 225 μL immobilized pH gradient (IPG)/ dithiothreitol (DTT) (4 μL 100× BioLyte 3/10 Ampholyte (Bio-Rad), 2 mg DTT (Sigma Aldrich) and 219 μL UT buffer).

Two-dimensional DIGE and gel analysis

Isoelectric focusing was carried out with some ReadyStrip IPG Strips pH 3–10 NL of 24 cm and a Protean i12 IEF Cell (Bio-Rad) during a passive rehydration of 9 h. The first dimension was carried out at 200 V for 2 h and the voltage was increased linearly to 1000 V over 4 h, then linearly to 10,000 V for 1 h and finally set at 10,000 V for 4 h and 30 min. The maximum current setting was 50 μA/strip in an isoelectric focusing unit. Following the first dimension, equilibration of the IPG strip was carried out according to the manufacturer's instructions (HPE-FlatTop Tower user manual; Serva Electrophoresis, Heidelberg, Germany). The IPG strips were incubated for 15 min in reduction solution (30% (w/v) urea, 83% (v/v) equilibration buffer, 0.83% (w/v) DTT), and then for a further 15 min in alkylation solution (30% (w/v) urea, 83% v/v equilibration buffer, 2% (w/v) iodoacetamide).

The second dimension electrophoresis was undertaken with the HPE FlatTop Tower (Serva Electrophoresis) on a 2D HPE Large Gel NF 12.5% acrylamide (Serva) according to the manufacturer's instructions. The preparative gel was placed overnight in a fixation buffer (10% acetic acid, 30% ethanol and 60% H_2O) and stirred. Gels were scanned with a Typhoon Ettan DIGE Imager (GE Healthcare, Freiburg, Germany) at wavelengths corresponding to each CyDye. The DIGE gel images were analyzed using Nonlinear Progenesis SameSpots (Nonlinear Dynamics, Newcastle upon Tyne, England, UK). Quantitative differences in spot intensity among the four treatment groups were analyzed by ANOVA implemented in the SameSpots 2D software version 3.5 Nonlinear Progenesis SameSpots. Differential regulation of proteins was compared by the log_2-fold-change approach.

Mass spectrometry

Spots of differential proteins were picked manually from the gel using a 1.5 mm picking pen (The Gel Company, San Fansisco, CA, USA). Gel pieces were washed twice with 50 mM NH_4HCO_3 and with a mix of NH_4HCO_3 (50 mM) and acetonitrile 50% (v/v). The proteins were then reduced with 10 mM DTT, alkyled with 55 mM iodoacetamide and washed twice as described previously. Gel pieces were dehydrated with 100% acetonitrile and finally digested for 5 h with trypsin. The peptide extraction was carried out with 1% (w/v) trifluoroacetic acid for 30 min at 40°C. The digested proteins were analyzed using a matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry UltrafleXtreme (Bruker Daltonics, Bremen, Germany).

Protein identification

The database used was the NCBI Database (restricted to Arthropoda taxonomies) and a homemade database containing aphid species and their known endosymbiont taxa. Searches on databases were treated with Bio-Tools 3.2 (Bruker Daltonics) on the Mascot server 2.2.06. Proteins were taken into account only when their score was of at least 45 and matched at least four peptides with error values <100 ppm.

Ability to colonize in the field

The reproductive performance of each clone separately was studied on naturally distributed plants: *Adesmia* sp. Hook. & Arn., *Astragalus germainii* Phil., *Lathyrus subandinus* Phil. and *Vicia nigricans* Hook. & Arn. These species were selected because they belong to plant genera that both clones were found to host, in central Chile (Peccoud *et al.* 2008). This study was carried out during the summer season of 2016 at the National Reserve Altos de Lircay (35°36′S, 71°00′W) in the Del Maule region, Chile. Two adult aphids of each clone were taken from the laboratory stock culture and were placed in clip-cages (2 cm diameter) on leaves of wild plants in their natural environment. Ten replicates per plant species and per clone were used. Plants were distributed over a linear transect of approximately 1.5 km. Ten days later, the clip-cages were revisited, and the total number of nymphs was counted, including alive and dead individuals. Data were analyzed with the generalized linear model with Poisson distribution and log link function with "clones" (pea and alfalfa genotypes) and "host" (the four species of wild legumes) as factors.

RESULTS

Genotyping and endosymbiont detection

Allele sizes of microsatellite loci are presented in Table S1. Six loci were common between this study and the first characterization of Chilean races of the pea aphid undertaken by Peccoud *et al.* (2008). Among these loci, *ALB12M* and *ApH10M* in Ap1 and *ALB07M* and *ApH10M* in Ap2 showed slightly different allele sizes between this study and the one of Peccoud *et al.* (2008). Thus, the samples collected from pea and alfalfa fields do not correspond exactly to multilocus genotypes described by Peccoud *et al.* (2008), although they are very similar. Therefore, our aphids sampled in pea and alfalfa fields will hereafter be named Ap1 and Ap2, respectively.

The presence of *Buchnera aphidicola* in both genotypes was also confirmed. Concerning the facultative endosymbionts, only *Serratia symbiotica* was found, and only in the Ap1 clone.

Ability to colonize in the laboratory

At both initial densities of nymphs (two and four), Ap1 reached a higher population growth rate than Ap2 (Fig. 1A,B). Indeed, a significant difference was observed between Ap1 and Ap2 with two initial individuals ($P < 0.001$), as well as with an initial density of four individuals ($P < 0.01$).

Feeding behavior

Among the dozens of calculated EPG parameters, only the total duration of single E1 was significantly different between the two genotypes ($P = 0.031$). Thus, Ap1
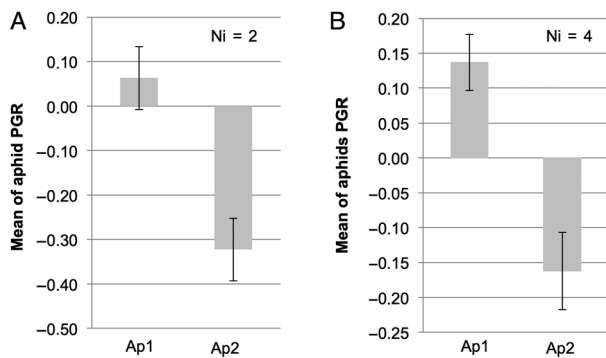
Figure 1 Comparison of population growth rate (PGR) (mean ± SEM) of *Acyrthosiphon pisum* between the two genotypes at similar initial density (*n* = 20). (A) For an initial density of two individuals (*P* < 0.001). (B) For an initial density of four individuals (*P* < 0.01).

performed longer events of salivation in phloem sieve elements, which were not followed by phloem ingestion phase (Fig. 2).

Protein identification

More than 100 proteins were differentially expressed between Ap1 and Ap2. According to our statistical threshold (*P* < 0.05, Student's *t*-test), a total of 54 spots showed significant differences between genotypes. Within these, 40 spots were identified and classified in different categories according to the biological function. Most of these proteins are related to cytoskeleton and energy metabolism (Table 1).

Fourteen proteins were upregulated in the Ap1 clone. The protein group showing the highest number of upregulated proteins in Ap1 was associated with
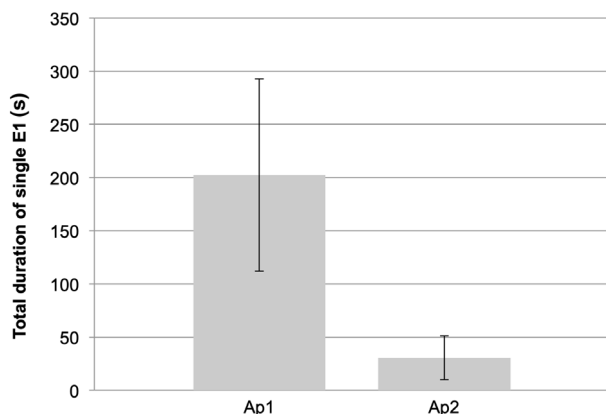
Figure 2 Total duration (mean ± SEM) of single events of salivation in phloem (E1) for Ap1 (*n* = 10) and Ap2 (*n* = 12) genotypes of *Acyrthosiphon pisum*, recorded with an electrical penetration graph over 8 h.

cytoskeleton (5 proteins). Among them, the actin partial (spot 6288) was the most upregulated with 1.7-fold. In comparison, 26 proteins were upregulated in the Ap2 clone. Energy metabolism was the protein group showing the highest number of upregulated proteins (9 proteins) in Ap2. Among them, the V-type proton ATPase catalytic subunit A-like was the most upregulated with 1.8-fold (spot 6424).

Ability to colonize in the field

Ap1 and Ap2 clones showed variation in the number of nymphs produced on different wild plants (clone × host interaction: $F_{3,70}$ = 13.4, *P* < 0.01). Both clones were able to colonize wild legumes, performing better on *V. nigricans* (Fig. 3). However, on this plant, Ap1 produced a significantly higher number of nymphs than Ap2 (main clone effect: $F_{1,76}$ = *P* < 0.05). In contrast, Ap2 produced more nymphs than Ap1 on *As. germainii*. Although not significantly, Ap2 exhibited higher reproduction than Ap1 on the other two wild legumes (Fig. 3).

DISCUSSION

On ability to colonize and probing behavior

The study of population growth rate showed that the Ap1 clone described herein shows higher ability to colonize broad bean than the Ap2 clone. This agrees with the study of Peccoud *et al.* (2008) where the performance of Ap1 clones was globally higher than Ap2, because Ap1 is specialized to the Vicieae tribe. It was also confirmed in our field experiment, as Ap1 showed better performance on the wild *V. nigricans*. Surprisingly, in our study, Ap2 exhibited a trend to perform better on the three wild legumes tested, especially on *As. germainii*, suggesting higher ability to colonize wild plants. This is not in agreement with the former description of Ap1 and Ap2 in Chile, reported by Peccoud *et al.* (2008), which showed Ap1 with wider host range than Ap2, particularly on wild plants. Nevertheless, the study of Peccoud *et al.* (2008) did not assess the performance of both clones by direct experiments on wild legumes, but drew their conclusions from the absence or presence of the genotypes during sampling. Interestingly, the differential performance of the clones on the wild legume *As. germainii* occurred despite several generations of laboratory rearing of both clones. Contradictory evidence in published reports makes it unclear whether the reproductive performance of host-specialized individuals is altered by aphid's experience on different host plants (Via 1991; Caballero *et al.* 2001; Liu *et al.* 2008; Ma *et al.* 2019),

Table 1 Differential proteins identified in both biotypes of *Acyrthosiphon pisum* (Ap1 and Ap2), average normalized volumes and related pathways

| Spot no.	Average normalized volume		Fold	Protein identification	NCBI accession	Mascot score	MS coverage	MW	pI-value
	Ap1	Ap2							
Stress tolerance									
3260			1.2	T-complex protein 1 subunit eta-like (*Acyrthosiphon pisum*)	gi|193 577 789	119	39	59 872	6.6
6483			1.2	Heat shock 70 kDa protein F, mitochondrial-like (*Acyrthosiphon pisum*)	gi|641 653 643	118	35	75 300	9.4
4619			1.6	Chaperonin GroEL, partial (*Buchnera aphidicola*)	gi|429 474 333	86	20	36 122	5.1
4681			1.6	Symbionin (*Buchnera aphidicola*)	gi|5660	94	22	57 989	4.9
Cytoskeleton									
3963			1.3	Actin related protein 1 (*Acyrthosiphon pisum*)	gi|217 330 650	104	40	42 158	5.2
5672			1.6	Actin-1, partial (*Acyrthosiphon pisum*)	gi|239 792 566	87	43	12 794	5.3
6288			1.7	Actin, partial (*Acyrthosiphon pisum*)	gi|169 218 603	71	50	22 414	5.04
6515			1.4	Actin-1, partial (*Acyrthosiphon pisum*)	gi|641 663 835	88	59	12 794	5.3
6516			1.4	Actin-1, partial (*Acyrthosiphon pisum*)	gi|641 663 835	51	33	12 794	5.3
6418			1.3	Putative actin (*Acyrthosiphon pisum*)	gi|89 473 786	80	15	42 194	5.19
6416			1.2	Tubulin beta-1 (*Acyrthosiphon pisum*)	gi|298 676 439	147	55	50 637	4.72
6417			1.3	Tubulin alpha chain-like (*Acyrthosiphon pisum*)	gi|193 594 183	145	55	50 550	5.01
Amino acid metabolism									
3487			1.8	Glutathione synthetase-like isoform X2 (*Acyrthosiphon pisum*)	gi|193 603 332	88	28	54 817	6.2
6458			1.4	Gamma-glutamylcysteine synthetase, putative (*Acyrthosiphon pisum*)	gi|240 848 683	50	11	28 815	5.06
3420			1.3	Alanine aminotransferase 2 isoform X2 (*Acyrthosiphon pisum*)	gi|641 656 881	82	14	54 327	5.9
Energy metabolism									
3255			1.6	Pyruvate kinase-like isoform 1 (*Acyrthosiphon pisum*)	gi|193 624 987	223	53	56 526	6.7
4405			1.3	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial-like (*Acyrthosiphon pisum*)	gi|328 714 666	107	44	39 545	5.8
3638			1.4	Probable citrate synthase 2, mitochondrial (*Acyrthosiphon pisum*)	gi|193 676 562	109	35	52 307	7.8
3821			1.4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 2 (*Acyrthosiphon pisum*)	gi|641 675 606	50	23	10 403	9.55
3892			2.2	Probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial-like (*Acyrthosiphon pisum*)	gi|193706910	117	42	46 309	9.2
3927			1.6	Acetyl-CoA acetyltransferase, cytosolic-like (*Acyrthosiphon pisum*)	gi|328 723 444	81	27	41 629	6.4
3963			1.3	ACYPI006035 (= 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial-like) (*Acyrthosiphon pisum*)	gi|239 790 994	82	30	41 480	6.28
6424			1.8	V-type proton ATPase catalytic subunit A-like (*Acyrthosiphon pisum*)	gi|193 690 627	107	31	68 057	4.8
6439			1.3	Putative ATP synthase beta subunit (*Acyrthosiphon pisum*)	gi|89 574 469	211	62	55 778	4.8

(*Continues*)

Table 1 Continued

Spot no.	Average normalized volume		Fold	Protein identification	NCBI accession	Mascot score	MS coverage	MW	pI-value
	Ap1	Ap2							
6442			1.4	ATP synthase subunit beta, mitochondrial (*Acyrthosiphon pisum*)	gi|209 915 626	186	69	55 777	4.9
Protein processing									
5277			2.1	Elongation factor 1-alpha (*Acyrthosiphon pisum*)	gi|641 662 724	84	18	50 278	9.3
6491			1.3	Peptidase (*Buchnera aphidicola*)	gi|503 643 941	65	31	89 552	9.69
4621			1.4	Cathepsin B-348 (*Acyrthosiphon pisum*)	gi|157 058 763	61	23	28 084	6.34
4103			1.4	Golgin subfamily A member 7 (*Acyrthosiphon pisum*)	gi|648 215 779	55	21	16 984	8.52
Signalling pathway									
4725			1.3	14–3-3 protein zeta (*Acyrthosiphon pisum*)	gi|251 823 883	120	52	28 322	4.6
6404			2	Tyrosine-protein phosphatase non-receptor type 23 (*Acyrthosiphon pisum*)	gi|193 706 873	224	58	62 983	8.9
6406			2.1	Tyrosine-protein phosphatase non-receptor type 23 (*Acyrthosiphon pisum*)	gi|193 706 873	200	49	62 983	8.9
6452			1.7	Putative nuclear envelope pore membrane protein POM 121B (*Acyrthosiphon pisum*)	gi|193 620 175	151	39	72 602	8.3
RNA polymerase transcription									
4586			1.3	Transcription elongation factor GreA (*Serratia symbiotica*)	gi|217 330 650	70	41	17 767	4.82
Other function									
1770			2.2	Uncharacterized protein LOC103310553 (*Acyrthosiphon pisum*)	gi|641 649 900	54	20	31 842	9.3
4692			1.7	ACYPI007550 (*Acyrthosiphon pisum*)	gi|239 791 062	50	24	24 815	7.59
4924			1.3	Apolipoprotein D (*Acyrthosiphon pisum*)	gi|641 649 326	50	12	24 799	9.18
6456			1.6	ACYPI001858 (*Acyrthosiphon pisum*)	gi|239 788 077	45	11	37 360	8.88

Average volume normalized: 0.4 — 2.4

The color scale represents the variations of expression: the higher the expression, the brighter the red. MS coverage, sequence coverage as the percentage of protein sequence covered by observed peptides (%); Fold, maximum differential expression factor between two treatments; Mascot score, Mowse score according to Mascot search; MW, molecular weight of the protein (Da); NCBI, National Center for Biotechnology Information; pI, isoelectric point.
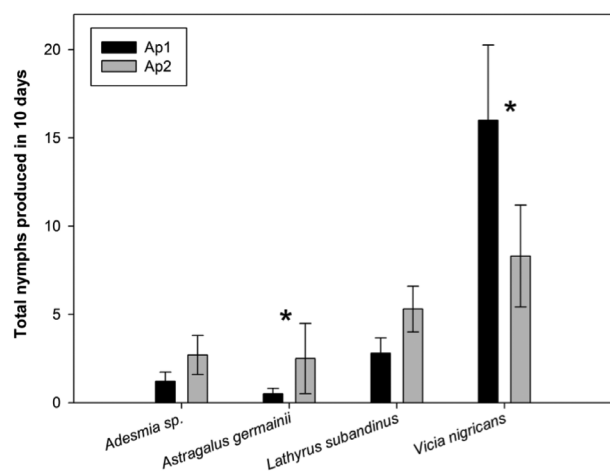
Figure 3 Total number (mean ± SEM) of nymphs produced by Ap1 and Ap2 clones of *Acyrthosiphon pisum* in 10 days in the field, starting from two adult individuals (n = 10). An asterisk (*) indicates significant difference ($P < 0.05$) between clones on one host plant.

although evidence points that experience modifies performance in individuals with wider diet breadth (Olivares-Donoso *et al.* 2007; Slater *et al.* 2019).

Our field experiment showed a weakness related to the low production of nymphs. This could be due to the short period of time attributed to this experiment and to the fact that aphids were confined inside clip-cages. However, caged aphids on *V. nigricans* produced between three and eight times more nymphs than aphids on the other wild leguminous plants. Therefore, despite the low values of our results, we were able to statistically highlight differential fecundity of the aphid clones between wild leguminous plants. Moreover, low reproduction of aphids would be minimalized by the exponential population growth rate frequently observed for aphids. On the contrary, a small change in the initial density of colonizing colonies inflicts important consequences on the population dynamics of aphids (Kindlmann & Dixon 2010).

Remarkably few probing behavior parameters were significantly different between the clones, which suggests that both clones were able to feed successfully on *V. faba*. Therefore, a lack of food ingestion cannot explain the low development of Ap2. However, we suggest that Ap1 is particularly well adapted to this host plant, being able to modify its salivation process in phloem, a key step in plant–insect interactions (Will *et al.* 2007; Harmel *et al.* 2008; Giordanengo *et al.* 2010). In *A. pisum* saliva, several effector proteins have been identified that modulate aphid feeding and survival (Wang *et al.* 2015a, 2015b) and potentially inhibit plant defense responses in phloem (Will

et al. 2007; Naessens *et al.* 2015). Finally, the presence of secondary endosymbionts can impact the salivary compounds of aphids (Su *et al.* 2015). Then, if salivation phases are enhanced because of longer duration of single E1 events, the quantity of effectors injected into the host plant is impacted and aphids would perform better on host plants. Modification of probing behavior due to varying plant suitability deserves more interest from EPG researchers.

On aphid endosymbionts

The Ap1 clone harbored the secondary endosymbiont *S. symbiotica*. This is coherent with the results of Sepúlveda *et al.* (2017), who also found clone Ap1 harboring *S. symbiotica* in high prevalence in Chilean field populations of *A. pisum*. However, detection of secondary endosymbionts in Ap2 clones was different from the study of Sepúlveda *et al.* (2017) in which *H. defensa* and *R. insecticola* were also found. Differences between field and laboratory detections could result from a combination of geographical variation (Sepúlveda *et al.* 2017) and the loss of endosymbionts as a consequence of long-term rearing in the laboratory (Desneux *et al.* 2018). In Europe, the association between *S. symbiotica* and aphids on *P. sativum* is also one of the most frequent in field conditions (Frantz *et al.* 2009; Ferrari *et al.* 2011; Henry *et al.* 2013). It is worth noting that *Serratia*-positive pea aphid lines have been found more susceptible to insecticides than non-infected lines (Skaljac *et al.* 2018). *Serratia symbiotica* provides protection against heat stress, but shortens the time taken by the aphid to complete its development from larva to adult (Russell & Moran 2005; Laughton *et al.* 2014). Thus, the prevalence of *S. symbiotica* in the Chilean Ap1 is coherent with the climate conditions of central Chile, with mild winter conditions and warm summer, which are features favoring the expansion of aphid asexual linages (Figueroa *et al.* 2018).

On aphid proteome

Aphids could adapt to less suitable plants by a series of metabolic changes, regulated by differential expression of proteins (Lu *et al.* 2016) along with adaptive processes. Here, our study focused on the whole-body proteomic profile variation among the two predominant genotypes of *A. pisum* in Chile to better understand the adaptation process and explain the dominance of a clone (Ap1) compared to the others. Our results highlight some differences between the clone specialized on *P. sativum* (Ap1) and the one specialized on *M. sativa* (Ap2). Indeed, the proteomic analysis allowed the

isolation of more than 100 differential protein spots. The 40 identified proteins have been classified into eight groups according to their function: stress tolerance (10%), cytoskeleton (25%), amino acid metabolism (8%), energy metabolism (25%), protein processing (10%), signaling pathway (10%), RNA polymerase transcription (3%) and other functions (10%).

Stress tolerance

Contrary to their name, molecular chaperones, including heat shock proteins (HSPs), are not only produced under stress conditions but also in normal conditions (Pockley 2003). Indeed, they are known to play diverse functions, like facilitating protein folding and stabilization as well as disassembly and degradation of damaged or aggregated proteins (Morimoto et al. 1994; Hartl 1996; Feder & Hofmann 1999). Here, aphid and bacterial proteins were differently expressed in both genotypes. For example, HSP60 (T-complex protein) and HSP70 were upregulated in the most specialist clone, Ap2; they bind to partly folded or to extended polypeptides, respectively (Pockley 2003). Sørensen et al. (2003) suggested that the expression level of HSPs is a balance between benefits and costs: an overexpression of HSPs leads to a negative impact on growth rate and fertility. This could partially explain the higher population growth rate for Ap1 clone than Ap2 in the laboratory. The second group of proteins was represented by chaperonin GroEL and symbionin, both upregulated in clone Ap1. Symbionin is the original name of the GroEL protein and represents the most abundant protein in *Buchnera* (Ishikawa & Yamaji 1985; Baumann et al. 1996). It would seem that *Buchnera* bacteria is in lower quantity in Ap2 than in Ap1. It could be interesting to undertake a quantitative PCR to confirm this hypothesis. Fares et al. (2002) described the very important role of GroEL in the functional maintenance of the endosymbiont proteome. Moreover, they confirmed the hypothesis made by Moran (1996) and proposed that the evolution of this protein is governed by positive selection. This could give an ecological advantage to clone Ap1.

Cytoskeleton

Actin microfilaments, intermediate filaments and rigid microtubules compose the cytoskeletal network. Actin is a highly conserved protein that acts on the contractile machinery leading to cellular and muscular movements (Francis et al. 2006). Tubulin is involved in many cellular functions such as cell division, intracellular exchanges and growth of neurons. Variations of tubulin production in aphids have consequences on

formation of all microtubules (Hachouf-Gheras et al. 1998; Kawasaki et al. 2003) and thus, disturbing the peripheral nervous system (Bo & Wensink 1989; Kawasaki et al. 2003). Our results show that most actin-related proteins were upregulated in Ap1. In contrast, tubulin-related proteins (α and β) were upregulated in Ap2. Francis et al. (2010) advanced the hypothesis that modifications of the insect cytoskeleton represent an adaptation to a variety of stresses, including exposure to host plant defenses. Moreover, chaperonins containing TCP-1 (CCP) stabilize quasinative structures in both proteins, suggesting their involvement in protein folding. Nevertheless, CCP could interact preferentially with tubulin rather than actin (Llorca et al. 2000). This agrees with our results because CCP and tubulin-related proteins were upregulated in clone Ap2.

Amino acid metabolism

The production of glutathione (GSH) is catalyzed by the consecutive action of two enzymes: glutathione synthetase (GS) and γ-glutamylcysteine synthetase (GCS). Both belong to the γ-glutamyl cycle involved in some critical functions: protection of cells from oxidative damage, transport of amino acid, detoxification of xenobiotics and as a cofactor for a number of enzymes. Glutathione synthetase catalyzes the formation of GSH, and GCS is inhibited by GSH (Anderson 1998; Polekhina et al. 1999). In our study, GS is upregulated in clone Ap1, whereas GCS is upregulated in clone Ap2. Interestingly, the upregulation of GCS observed in clone Ap2 leads to an overproduction of 5-oxoproline, leading to metabolic acidosis (Kortmann et al. 2008).

Alanine aminotransferase is involved in arginine biosynthesis and is upregulated in clone Ap2. According to the International Aphid Genomic Consortium (IAGC 2010), the synthesis of arginine by the pea aphid is impossible due to a lack of genes. Nevertheless, *A. pisum* and *Buchnera* are broadly complementary for amino acid biosynthesis. So, the gene for alanine aminotransferase could be highly expressed in the bacteriocytes harboring *Buchnera* and therefore collaborate in arginine synthesis.

Energy metabolism

The identified proteins are involved in the tricarboxylic acid cycle, glycolysis, mitochondrial biogenesis, fatty acid metabolism, oxidative phosphorylation, and valine, leucine and isoleucine degradation. All proteins were upregulated in clone Ap2 excepting the medium-chain specific acyl-CoA dehydrogenase. This is implicated in the valine, leucine and isoleucine degradation

pathway and fatty acid metabolism (Kanehisa *et al.* 2016). It can be assumed that the metabolism of clone Ap2 is comparatively more dynamic than Ap1, with more upregulation of key proteins that, in the long term, leads to a cost in terms of reproduction.

Protein processing

A peptidase involved in the maturation process of proteins was identified. This protein is related to the maturation process of proteins but also in plant–aphid interactions by cleaving host defense proteins (Vandermoten *et al.* 2013). The Golgin subfamily A member 7 (Golga7) might be involved in protein transport from Golgi to cell surface (The UniProt Consortium 2014). Both proteins are upregulated in clone Ap2.

In the group of protein processing, the only protein upregulated in clone Ap1 was cathepsin B-348, a lysosomal cysteine protease. Through aphid lineage, the evolution was found to select the amplification of cathepsin B to counteract the poverty of their diet (Rispe *et al.* 2008). Indeed, aphids feed on phloem, which is deficient in 10 essential amino acids (Douglas & van Emden 2007). The prevailing idea is that the obligate bacterial endosymbiont, *B. aphidicola*, supplies and compensates the unbalanced diet (Douglas & van Emden 2007; Oliver *et al.* 2010). Nevertheless, symbiosis would not be the only way to improve its feeding. In fact, there is a growing body of evidence showing that cathepsin B is used by the aphid to manipulate plant defense reactions (Rispe *et al.* 2008; Giordanengo *et al.* 2010).

Signaling pathway

The 14-3-3 protein zeta, involved in the signaling pathway, was upregulated in clone Ap1. Highly conserved and ubiquitous, the family of 14-3-3 proteins is able to bind a variety of proteins. Interestingly, they are involved in essential cellular processes such as signal transduction, cell cycle regulation and stress response (Fu *et al.* 2000; van Hemert *et al.* 2001). Furthermore, the activation of signaling proteins requires the interactions of 14-3-3 protein with these proteins (Muslin *et al.* 1996). This could give an advantage to clone Ap1, for example, by anticipating adverse effects of plant defense.

Other functions

It was found that overexpression of apolipoprotein D (apoD), upregulated in Ap1, increases resistance to oxidative stress and starvation, contributing to lifespan expansion in fruit flies (Sanchez *et al.* 2006; Walker *et al.* 2006). Finally, the uncharacterized protein LOC103310553 possessed the most important fold change ratio. Knowing its function will perhaps help to further understand the mechanisms of adaptation between aphid and plant.

CONCLUSIONS

To conclude, in our study, we confirmed that Chilean clones of *A. pisum*, specialized on pea or alfalfa, are genetically divergent and that each clone possesses a specific distribution of facultative endosymbionts. A *Serratia*-positive aphid line was significantly more susceptible to exposed insecticides than a non-infected line. The population growth rate showed that Ap1 seems to be better adapted to the broad bean plant than Ap2, confirming specialization to this host. The field experiment also confirmed the ability of these clones to reproduce on wild legumes. Comparatively, clone Ap1 presented lower alteration of its physiology, but both genotypes showed adaptations to manipulate plant defense. The metabolism of clone Ap2 seemed to be more dynamic than that of Ap1, with more upregulated key proteins that, in the long term, seems to drive reproductive costs. Because all experiments were carried out on laboratory-reared strains, more studies should be undertaken with natural aphid populations to confirm our results.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1. Microsatellite genotyping of Ap1 and Ap2 *Acyrthosiphon pisum* clones